Biochimica et Biophysica Acta 1402 (1998) 250–260

A chemically modified tetracycline inhibits streptozotocin-induced diabetic depression of skin collagen synthesis and steady-state type I procollagen mRNA

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Received 20 October 1997; revised 5 January 1998; accepted 6 January 1998

Abstract

Wasting of connective tissues including skin, bone, and cartilage have been closely associated with elevated matrix metalloproteinase (MMP) activity and depressed collagen content in the streptozotocin (STZ)-induced diabetic rat, while tetracyclines have been reported to normalize total body weight, skin hydroxyproline and collagen content in this model, in part through inhibition of MMPs. In the present study, we report the effect of CMT-1, a chemically modified tetracycline that lacks antimicrobial properties but retains divalent cation binding and MMP inhibitory activity, on diabetic skin collagen synthesis and steady-state levels of procollagen $\alpha 1$(I) mRNA. Male, 4-month old Sprague–Dawley rats received a single injection of 75 mg/kg STZ or citrate vehicle alone and diabetic status was confirmed by positive glucosuria. Some diabetic animals received 10 mg/day of CMT-1 by oral gavage and, 28 days after STZ treatment, body weight, blood glucose values and the in vivo rates of skin collagen production were measured using the pool-expansion technique. Steady-state levels of procollagen $\alpha 1$(I) mRNA were analyzed 21 days after STZ treatment by hybridization of total RNA with a ^{32}P labelled cDNA to rat type I procollagen $\alpha 1$(I) mRNA in a dot-blot assay. STZ treatment was found to significantly depress body weight, skin collagen hydroxyproline content, the in vivo rate of collagen production, and hybridizable levels of type I procollagen $\alpha 1$(I) mRNA. CMT-1 administered daily to STZ-treated rats inhibited the diabetic depression of these parameters but had little or no effect on non-diabetic controls or on STZ-induced hyperglycemia. Thus, in addition to the inhibition of MMP mediated extracellular collagen degradation, these results suggest CMT-1 also acts to inhibit diabetic connective tissue breakdown in STZ-induced diabetes by increasing both steady-state levels of type I procollagen mRNA and collagen synthesis through mechanism(s) that are independent of the antibacterial properties of tetracyclines. © 1998 Elsevier Science B.V.

Keywords: Collagen; Diabetes; mRNA; Tetracycline

1. Introduction

Wasting of connective tissues has historically been a prominent feature of uncontrolled insulin-dependent

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PII S0167-4889(98)00008-1

diabetes mellitus. In the streptozotocin (STZ)-induced diabetic rat, atrophy of connective tissues including skin [1,2], bone and cartilage [3,4] have been closely associated with depressed collagen content. Since the total tissue collagen mass is the result of both rates of formation and degradation, depressed connective tissue collagen content associated with STZ-induced diabetes may result from increased collagen degradation and/or depressed levels of total collagen synthesis. Indeed, increased skin collagenase activity and excessive skin collagen degradation have been reported in the STZ-induced diabetic rat [2,5] and increased collagen degradation has been proposed as a possible mechanism for depressed accumulation of newly synthesized collagen observed in this model [6]. However, direct depression of collagen synthesis in the STZ-induced diabetic rat has also been reported for several connective tissues including skin [7], gingiva [8] and bone [9,10]. These results suggest that depressed collagen content observed in the STZ-induced diabetic rat may be the result of multiple factors active at several regulatory points.

Tetracyclines have been reported to inhibit excessive connective tissue degradation in several pathologic processes including STZ-induced diabetes [6,11–13]. Tetracyclines are known to be potent inhibitors of extracellular matrix metalloproteinases (MMPs), including the three collagenases MMP-1, MMP-8 and MMP-13, and two gelatinases MMP-2 and MMP-9 [14,15], and are thought to act, in part, through the chelation of divalent cations required as MMP cofactors [16,17]. It is therefore possible that tetracyclines may increase total collagen production in the STZ-induced diabetic rat through the inhibition of extracellular MMPs [11] and intracellular proteolysis [18]. In support of this hypothesis, doxycycline, minocycline, and 4-dedimethylaminotetracycline, a chemically modified tetracycline lacking antimicrobial properties but retaining divalent cation binding, have all been reported to inhibit intracellular myoblast proteolysis in vitro [18], perhaps through inhibition of Ca^{2+} and Zn^{2+} dependent proteinases (myosinase) [19]. This effect of tetracycline may have relevance to diabetes-induced wasting in muscle tissue as well.

However, several studies have also reported direct anabolic effects on collagen synthesis by tetracyclines in the STZ-induced diabetic rat. Tetracycline treatment of diabetic rats resulted in increased total body weight and skin collagen mass [11,12], incorporation of [^{3}H]proline into periodontal ligament fibroblasts [20], total skin RNA content, and the in vivo rate of collagen production [12]. Taken together, these results suggest that tetracyclines act at several sites to inhibit the loss of collagen in various tissues during poorly controlled/uncontrolled diabetes, specifically through inhibition of collagen degradation and stimulation of collagen synthesis.

The present study was designed to further characterize the anabolic effect of tetracyclines on skin collagen synthesis in the STZ-induced diabetic rat. We first addressed whether the prevention of the loss of skin collagen in the STZ-induced diabetic rat was due to the antibiotic or divalent ion chelation property of tetracyclines by administration of a tetracycline derivative lacking antimicrobial activity but retaining divalent cation binding. Then, through analysis of in vivo rates of skin collagen production and steady-state levels of type I procollagen mRNA, we have begun to define the site in the biosynthetic pathway at which tetracyclines may act to inhibit STZ-induced depression of skin collagen synthesis. Our results suggest that tetracyclines inhibit the STZ-induced diabetic depression of skin collagen synthesis, in part, through a non-antimicrobial mechanism, at a pre-translational site by enhancing steady-state levels of type I procollagen mRNA.

2. Materials and methods

2.1. In vivo protocol

Four month old male Sprague–Dawley rats (Charles River Breeding Laboratories, Cambridge MA) received, on day 0, an intravenous injection via the tail vein of streptozotocin (2-deoxymethyl nitrosourea glucopyranose, STZ, Sigma, St. Louis, MO, 75 mg/kg body weight) dissolved in isotonic saline and 0.1 M citrate buffer (pH 4.5) (diabetic group) or isotonic saline and 0.1 M citrate buffer alone (control group). Diabetic status was verified using a test tape for urine glucose (Eli Lilly). Some STZ treated animals, beginning on day 0, also received 10 mg/day (for measurement of collagen protein synthesis) or 5 mg/day (for measurement of steady state levels of type I procollagen mRNA) of a chemically-modified tetracycline (4-dedimethylamino tetracycline, CMT-1) in an aqueous 2% (w/v) carboxymethyl cellulose suspension delivered by oral intubation (diabetic + CMT group). CMT-1 was synthesized according to McCormick et al. [21] and Boothe et al. [22] and is a tetracycline derivative lacking antimicrobial activity but retaining its cation binding site at carbon-11 and carbon-12 and associated MMP inhibitory activity [11,17]. Animals were maintained for 28 days and given Purina Laboratory Rat Chow (Ralston Purina, St. Louis, MO) and water ad libitum.

2.2. Measurement of collagen protein synthesis

The pool expansion approach [8] was used to determine the effect of STZ induced diabetes and CMT treatment on total skin collagen production. Two in vivo labeling protocols were followed using different concentrations of [^{3}H]proline. Twenty-eight days after STZ treatment, some animals received intraperitoneal injections of 2 mCi of [2,3 ^{3}H]proline (New England Nuclear, Cambridge, MA) in 2.0 ml of isotonic saline containing 800 mg L-proline and were sacrificed 0.5 h later to measure intracellular procollagen pools. Some animals received intraperitoneal injections of 1 mCi of [2,3 ^{3}H]proline (New England Nuclear, Cambridge, MA) in 2.0 ml of isotonic saline containing 800 mg L-proline and were sacrificed 2 h later to determine the effect on total collagen production including the recently secreted extracellular fraction. At completion of either labeling period, blood was collected by intra-cardiac puncture after halothane anesthesia induction. Serum glucose was determined using a glucose oxidase assay kit (Sigma, St. Louis, MO). Skin from the entire torso was removed as previously described [23] and weighed.

Skin hydroxyproline (an amino acid marker of collagen) and proline content were determined by extracting 1.5 g (wet weight) of minced, defatted skin in 10% trichloroacetic acid (TCA) to yield TCA soluble and insoluble fractions. The TCA soluble fraction was lyophilized, dissolved in 2 ml of 1 M ammonium acetate and the proline content determined by the tandem column procedure as previously reported [24]. The TCA insoluble fraction was hydrolyzed in 6 M HCl at 105°C and the released [^{3}H]proline and [^{3}H]hydroxyproline isolated using the tandem column procedure. The prolyl-tRNA pool was isolated and measured essentially as described by Schneir et al. [12]. In brief, 6.0 g (wet weight) of tissue was homogenized in 30 ml of cacodylic buffer (50 mM cacodylic acid, 1% sodium dodecyl sulfate, pH 6.0) using a polytron (Brinkman Instruments, Luzern, Switzerland). The homogenate was extracted in one volume phenol/cacodylic buffer (88:12, v/v), the aqueous fraction recovered, and nucleic acids precipitated at −20°C overnight by the addition of 2.5 volumes of 95% ethanol and collected by centrifugation ($10\,000 \times g$ for 15 min at 4°C). The pellet was resuspended in 0.5 M ammonium hydroxide, incubated at 37°C for 1 h and ethanol precipitated as described before. The pellet was lyophilized, dissolved in 2 ml of 1 M ammonium acetate and processed for the isolation of proline as described above. Skin collagen mass, relative collagen production, fractional rate of collagen production and absolute rate of collagen production were calculated from the above data as described by Schneir et al. [12].

2.3. Measurement of steady state levels of type I procollagen mRNA

Total RNA was isolated using a modification of the method of Chomczynski and Sacchi [25]. All reagents were molecular biology grade and the usual precautions against RNAse were followed including the treatment of all solutions with 0.1% diethylpyrocarbonate overnight followed by autoclaving. Phenol was obtained from Boehringer Mannheim (Indianapolis, IN) while all other regents unless specified were obtained from Fisher Scientific (Piscataway, NJ).

Immediately after sacrifice, the rat skin was weighed, minced and immersed in liquid nitrogen. Five grams of frozen tissue was added to 12.5 ml denaturing solution (4 M guanidinium isothiocynate, 25 mM sodium citrate, 0.5% sarcosyl, 100 mM 2-mercaptoethanol, pH 7.0) and homogenized using a polytron in a sterile 50 ml capped centrifuge tube. Added in succession to the homogenate were 2.5 ml of 2 M sodium acetate (pH 4.5), 12.5 ml water-saturated phenol and 2.5 ml of chloroform/isoamyl alcohol (24:1, v/v). The suspension was mixed thoroughly for 10 s, placed on ice for 15 min and cleared by centrifugation at $10\,000 \times g$ for 20 min at 4°C.

The aqueous phase was recovered and transferred to a baked 30 ml Corex centrifuge tube, 1 volume of isopropanol added, and RNA precipitated at −20°C overnight. The precipitate was collected by centrifugation at $10\,000 \times g$ for 20 min at 4°C. The pellet was redissolved in 5.0 ml of denaturing solution and allowed to precipitate with 1 volume isopropanol at −20°C for 3 h. The precipitate was collected by centrifugation at $10\,000 \times g$ for 20 min at 4°C, the pellet washed once with cold 80% ethanol, and lyophilized. The pellet was dissolved in minimal double distilled sterile H_2O and total RNA quantitated by optical absorbance at 260.

A series of 1:2 dilutions starting from an initial 10 μg of total RNA was prepared and dotted onto a nylon membrane (Zeta Probe, BioRad Laboratories, Hercules, CA) immobilized in a vacuum manifold (Schleicher and Schuell, Keane, NH). The dot blot was baked under vacuum at 80°C for 1 h. The blot was prehybridized 6 h at 50°C in 12 ml of hybridization solution (50% deionized formamide, 4× SSPE (600 mM sodium chloride, 40 mM sodium phosphate, 4 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4), 1.0% sodium dodecylsulfate (SDS), 0.5% Blotto (10% Carnation non-fat dry milk in 0.025% sodium azide), 0.5 mg/ml sheared, denatured salmon sperm DNA) with constant shaking in a Dubnoff water bath in a customized polyethylene bag.

The blot was hybridized 12 h at 50°C in 12 ml of hybridization solution containing a ^{32}P labelled cDNA to the 3 prime triple helical domain of rat pro $\alpha 1$(I) collagen mRNA [26]. The insert was digested from the plasmid cloning vector by PST I, isolated on 0.8% agarose gels containing 0.5 μg/ml ethidium bromide, visualized by ultraviolet illumination, and recovered by continued electrophoresis onto a Whatman 3MM paper backed by dialysis membrane. The insert was eluted in extraction buffer (200 mM sodium chloride, 50 mM Tris–HCl, 1 mM EDTA), extracted with chloroform/phenol (1:1) and ethanol precipitated. The insert (10 ng) was labeled to greater than 6.8×10^8 cpm/μg DNA by random priming with T7 polymerase (PrimeIt, Stratagene, La Jolla, CA) in the presence of [α-^{32}P]deoxycytidine triphosphate (> 3000 Ci/mmol, Amersham, Arlington Heights, IL), as described by Feinberg and Vogelstein [27]. The labelled cDNA was denatured at 95°C for 5 min prior to addition to the hybridization bag. After hybridization, the blot was washed once in 2× SSPE, once in 2× SSPE in 0.1% SDS, and twice in 1× SSPE in 0.1% SDS. All washes were conducted at room temperature for 10 min with constant shaking. The hybridized blot was exposed to preflashed X OMAT AR film (Kodak, Rochester, NY) with enhancing screens at −80°C overnight.

The resulting autoradiograph was quantitated using an LKB Ultrascan laser densitometer and Pharmacia Gel Scan XC software (Pharmacia LKB Biotechnology, Piscataway, NJ). The relative level of hybridizable pro $\alpha 1$(I) collagen mRNA, reported as the treated/control ratio for each group, was determined by first plotting the optical density vs. the dilution for each dot, then calculating the slope of the linear portion of the resulting relationship and dividing the slope by that generated by the control as previously described [28]. Data was analyzed for statistical significance using a two tailed Student's t-test.

3. Results

STZ treatment markedly depressed total body weight and increased blood glucose levels, however, the daily administration of CMT-1 was able to inhibit the STZ-induced depression of body weight but not blood glucose values. As noted in Table 1, the diabetic group was markedly hyperglycemic and failed to gain, but on average lost, body weight 28 days after STZ treatment when compared to the sham-treated control group. The daily oral administration of 10 mg/day of CMT-1 to the STZ-treated group (diabetic + CMT-1) resulted in an increase in body weight without any significant effect on blood glucose levels when compared to the STZ treated (diabetic) group. Therefore, CMT-1, administered daily over the 28-day time period of the study, partially inhibited the loss in body weight associated with STZ-induced diabetes without any significant effect on STZ-induced hyperglycemia.

The failure to increase body weight observed 28 days following STZ treatment was associated with decreased skin collagen content (Table 2). Total skin collagen was estimated by measuring the hydroxyproline content in skin of 28-day control, diabetic, and diabetic + CMT-1 treatment groups. As reported

Table 1
Body weight and blood glucose levels of CMT-1 treated diabetic rats

Treatment group	Treatment day	(n)	Body weight (g)	Blood glucose (mg/dl)
Control	0	(5)	378 ± 11	98 ± 5
Control	28	(10)	474 ± 24	108 ± 3
Diabetic	28	(10)	322 ± 26^{a}	662 ± 9^{a}
Diabetic + CMT-1	28	(10)	391 ± 13^{b}	638 ± 9^{a}

Male, 4-month-old Sprague–Dawley rats received a single injection of 75 mg/kg STZ or citrate vehicle alone and diabetic status confirmed by positive glucosuria. Some diabetic animals received 10 mg/day of CMT-1 and 28 days after STZ treatment body weight and blood glucose were determined. Values are the mean ± S.D. for (n) animals.
[a] $p < 0.001$ vs. 28 day control.
[b] $p < 0.001$ vs. 28 day diabetic.

in Table 2, skin from the diabetic group contained 53% less hydroxyproline than the non-diabetic control animals on a per wet weight basis. However, skin from diabetic rats given 10 mg CMT-1 daily, contained 30% less hydroxyproline than 28-day controls but 50% more hydroxyproline than the diabetic group. Therefore, the daily administration of CMT-1 partially inhibited the decrease in total skin collagen content associated with STZ-induced diabetes and was correlated with the effect of CMT-1 on total STZ-induced diabetic body weight reported in Table 1.

Tetracyclines are known inhibitors of metalloproteinases [16] and excessive skin collagenase activity has been reported in the STZ-induced diabetic rat model [2,5]. However, to address whether CMT-1 also has direct, anabolic effects on diabetic collagen synthesis, as earlier studies using tetracyclines with antibiotic properties have suggested [12,20], levels of collagen protein synthesis were measured in vivo using [^{3}H]proline labeling and the pool expansion technique [8]. Twenty-eight days after STZ administration, animals received a single, intraperitoneal injection containing 2 mCi of [^{3}H]proline and 800 mg unlabeled proline. Two separate in vivo labeling intervals of 0.5 and 2 h were followed. The 0.5-h in vivo labeling period estimated intracellular collagen production, since collagen synthesized during this time interval has been reported to be primarily intracellular and thus protected from the action of extracellular proteases [29]. The 2-h in vivo labeling period was used to estimate the overall rate of newly synthesized skin collagen accumulation, both intracellular procollagen and recently secreted collagen.

Since insulin has well documented effects on connective tissue amino acid and hexose transport [30], we first measured the specific radioactivity of prolyl tRNA pools after STZ, or STZ + CMT-1 treatment. Total RNA was isolated from 6.0 g of frozen tissue and hydrolyzed by treatment with sodium hydroxide. The proline content of the hydrolysate was isolated by tandem column chromatography and quantitated

Table 2
Skin collagen mass of CMT-1 treated diabetic rats

Treatment group	(n)	Skin collagen (mg hydroxyproline)	% difference	
			vs. C	vs. D
Control	(8)	1044 ± 91		
Diabetic	(8)	491 ± 67	-53^{a}	
Diabetic + CMT − 1	(9)	736 ± 103	-30^{a}	$+50^{a}$

Values are the mean ± S.D. skin hydroxyproline content of 1.5 g of minced defatted skin of (n) animals 28 days after receiving 75 mg/kg of STZ, (diabetic), STZ + 10 mg/day CMT-1 (diabetic + CMT-1) or citrate vehicle alone (control) as described in Section 2.
[a] $p < 0.001$.

Table 3
Specific radioactivity of skin [^{3}H]prolyl tRNA pools

Treatment group	(n)	0.5 h label	(n)	2 h label
Control	(3)	3902 ± 392	(4)	1313 ± 210
Diabetic	(5)	4200 ± 462	(5)	1508 ± 179
Diabetic + CMT-1	(5)	2688 ± 2440	(5)	1197 ± 218

Values are the mean ± S.D. dpm [^{3}H]proline/μg proline for 6.0 g of skin from (n) animals 28 days after receiving 75 mg/kg of STZ, (diabetic), STZ + 10 mg/day CMT-1 (diabetic + CMT-1) or citrate vehicle alone (control). To determine the effect of diabetes and diabetes + CMT-1 treatment on skin collagen production, animals received 2 mCi of [^{3}H]proline 0.5 h or 1 mCi of [^{3}H]proline 2 h prior to sacrifice. The prolyl tRNA pool specific radioactivity was determined as described in Section 2.

by comparison of chromatographic peak heights with known standards. The radioactivity associated with the proline peak was then measured by liquid scintillation spectroscopy. As shown in Table 3, little difference was detected in the specific radioactivity of tissue prolyl tRNA pools in the 28-day control, diabetic, or diabetic + CMT-1 groups after either the 0.5 or 2-h in vivo labeling intervals. Therefore, any change in [^{3}H]proline incorporation into newly synthesized protein observed among the treatment groups of this study was not due to changes in the specific activity of the prolyl tRNA pools.

The in vivo rate of collagen production was measured using 1.5 g of minced skin extracted in 10% TCA, to remove unincorporated isotope, followed by acid hydrolysis. The liberated hydroxyproline and proline was isolated by tandem column chromatography, quantitated by comparison of peak heights with known standards, and the associated radioactivity measured by liquid scintillation spectroscopy. The values obtained were combined with the specific radioactivity of the prolyl tRNA pool and the absolute rate of skin collagen production calculated. The results are reported in Table 4. The levels of skin collagen synthesis in the diabetic group were approximately 10% of control values after both 0.5 and 2-h labeling intervals. However, skin collagen synthesis in diabetic rats treated with CMT-1 was not significantly different from that of the non-diabetic group. Therefore, these results suggest CMT-1 had direct anabolic effects on diabetic skin collagen synthesis in this model. Furthermore, CMT-1 appeared to act through a mechanism separate from the inhibition of extracellular metalloproteinases previously reported for tetracyclines [12,17], since the rate of skin collagen production in diabetic rats treated daily with CMT-1 was elevated to a similar level after both the 0.5 and 2-h labeling intervals.

In several systems, collagen production can be regulated, at least in part, through intracellular degradation of newly synthesized collagen protein [31] and in fact, intracellular procollagen degradation is enhanced during experimental diabetes [24]. However,

Table 4
Absolute rate of rat skin collagen production

Treatment group	(n)	[^{3}H]HYP + [^{3}H]PRO (dpm/skin $\times 10^{-3}$)	[^{3}H]prolyl tRNA (dpm/μmol $\times 10^{-3}$)	Absolute rate (μmol/h)
Control				
0.5 h	(3)	2394 ± 526	449 ± 45	21.2 ± 3.2
2 h	(4)	3310 ± 1195	151 ± 24	22.9 ± 10.9
Diabetic				
0.5 h	(3)	222 ± 65	474 ± 67	1.9 ± 0.8[a]
2 h	(5)	619 ± 151	173 ± 21	3.6 ± 1.1[a]
D + CMT-1				
0.5 h	(3)	1185 ± 750	368 ± 286	18.2 ± 13.5
2 h	(5)	2172 ± 555	137 ± 25	15.9 ± 3.3

Values are the mean ± S.D. for the radioactivity of the hydroxyproline plus proline pools, specific activity of the prolyl tRNA pool, and rate of collagen production for skin samples from (n) animals 28 days after receiving 75 mg/kg of STZ, (diabetic), STZ + 10 mg/day CMT-1 (diabetic + CMT-1) or citrate vehicle alone (control) as described in Section 2. Results after both 0.5 and 2-h in vivo labeling periods are presented.
[a] $p < 0.01$.

in most tissues, collagen synthesis is regulated proximally, at the level of transcription or translation [28,29]. To address the possibility that CMT-1 may stimulate collagen synthesis at a pretranslational site in the STZ-induced diabetic rat, we asked whether steady-state levels of type I procollagen mRNA were altered in the diabetic or diabetic + CMT-1 groups. Total skin RNA was isolated from diabetic, non-diabetic, diabetic + CMT-1, and non-diabetic + CMT-1 groups and quantitated by optical absorbance at 260 nm. A series of dilutions was prepared for each sample, starting from an initial 10 μg of total RNA, and dotted onto nylon membranes. The blot was hybridized with a ^{32}P-labelled cDNA to the 3 prime triple helical domain of rat pro α1(I) mRNA as described in Section 2, and the resulting autoradiograph of the hybridized RNA dot blot shown in Fig. 1.

The RNA dot blot was quantitated by optically scanning the dilution series for each sample. The optical density vs. the RNA dilution for each dot was plotted and the slope of the linear portion of the resulting relationship was compared to that of control. The relative levels of pro α1(I) mRNA, expressed as a percent of control levels, is presented for each of the four treatment groups in Fig. 2. Although variation in the mean level of hybridization was noted within each group, the diabetic group had approximately 30% of the level of hybridizable pro α1(I) mRNA seen in the non-diabetic groups. Treatment of diabetic animals with CMT-1 significantly increased hybridizable pro α1(I) mRNA to a level similar to that of non-diabetic controls, while in non-diabetic animals, daily CMT-1 treatment had little effect on hybridizable levels of pro α1(I) mRNA. Therefore, the stimulation in the rate of collagen protein synthesis observed in diabetic animals treated daily with CMT-1 (Table 4) correlated with increased steady-state levels of pro α1(I) mRNA (Fig. 2). These results imply that CMT-1 has direct

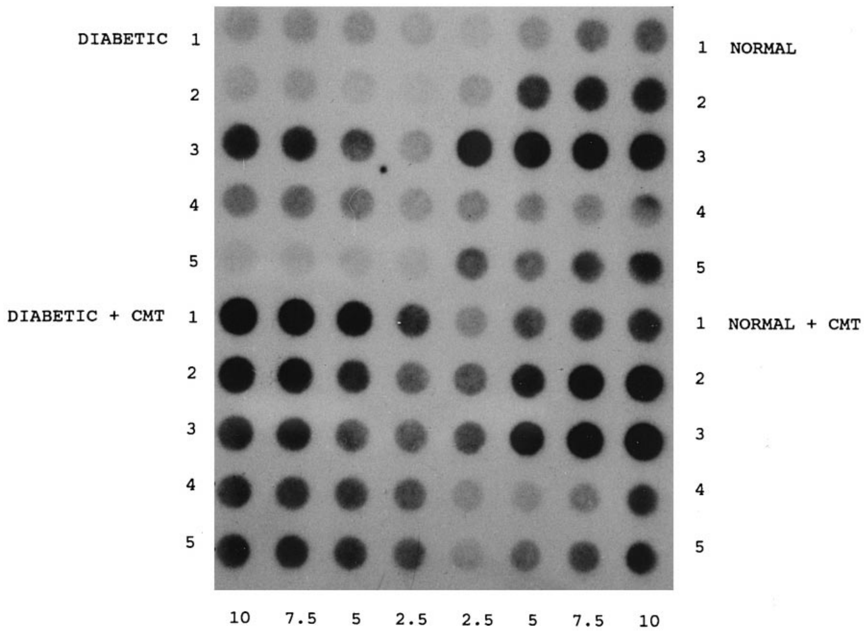

Fig. 1. Dot Blot of CMT-1 treated diabetic rat RNA hybridized with pα1R1. A series of four 1:2 dilutions starting with an initial 10 μg of total RNA from (n) animals 21 days after receiving 75 mg/kg of STZ, (Diabetic), STZ + 5 mg/day CMT-1 (Diabetic + CMT-1) or citrate vehicle or control alone was blotted onto a nylon membrane. The blot was hybridized with a ^{32}P-labelled cDNA to the 3′ triple helical domain of rat pro α1(I) mRNA [23], and subsequently exposed to X OMAT AR film with enhancing screens at −80°C overnight as described in Section 2.

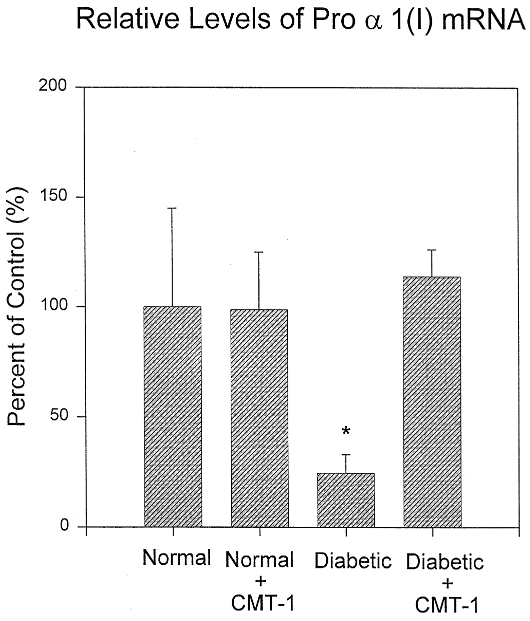

Fig. 2. Relative levels of pro α1(I) mRNA. The series of dilutions for each sample in Fig. 1 was optically scanned, the optical density vs. the dilution was plotted for each dot, the slope of the linear portion of the resulting relationship for each sample calculated and averaged within each group and compared to control. The mean ± S.D. for control (N), control + CMT-1 (N + CMT-1), diabetic (D), and diabetic + CMT-1 (D + CMT-1) is given as a percent of control (100%). * $p < 0.05$.

anabolic effects on diabetic skin collagen synthesis in the STZ-induced diabetic rat including the stimulation of steady-state levels of pro α1(I) mRNA.

4. Discussion

Treatment of 4-month old Sprague–Dawley rats with a single dose of STZ after 28 days resulted in the expected diabetic manifestations including total body weight loss, decreased skin collagen content and marked hyperglycemia; effects of STZ well documented in previous reports [1–3,9,11,12,23,24]. The oral administration of 10 mg/day CMT-1 inhibited the STZ-diabetes induced loss in body weight and decreased skin collagen content without significant inhibition of STZ-induced hyperglycemia. Thus, CMT-1, a tetracycline derivative without antibiotic properties but retaining divalent cation binding [11], was able to replicate results of earlier studies which reported inhibition of connective tissue wasting in STZ-induced diabetic adult rats by tetracyclines with antibiotic properties [11,12,20]. The results of the present study, together with those using germ-free animals [6], suggest that inhibition of STZ-induced diabetic connective tissue wasting by tetracyclines is not mediated by antibiotic but rather other properties of tetracyclines, possibly involving the chelation of divalent cations.

While the inhibitory effects of tetracyclines on excessive collagen degradation associated with STZ-induced diabetes have been well documented [6,11,17] and act presumably through divalent cation chelation [11] and inhibition of host-derived matrix metalloproteinases [17], the present study was specifically designed to address the anabolic effects of tetracyclines on diabetic skin collagen metabolism. It is of interest to note that, in the present study, CMT-1 was unable to completely reverse the STZ-induced loss of body weight and skin collagen mass. These results are in agreement with other studies of tetracyclines in STZ-induced diabetes [11,12]. Although it is possible that incomplete reversal of STZ-induced weight loss and associated skin collagen content could have resulted due to the lack of pair feeding or the use of sham intubation controls for daily CMT-1 oral administration, it is more likely that tetracyclines are not direct antagonists of the diabetogenic effects of STZ but rather exert their anabolic effects on skin collagen synthesis through mechanisms distinct from inhibition of STZ-induced hyperglycemia and hypoinsulinemia.

Along these lines, a primary finding of the present study was that the reduction of total skin collagen content and suppression of skin collagen synthesis in the diabetic rat were both inhibited by the daily oral administration of CMT-1. These results agree with prior studies using tetracyclines with antibiotic properties [11,12,20]. The increased levels of skin collagen synthesis observed in the diabetic + CMT-1 group, when compared to the diabetic group alone, was apparently not due to changes in the specific activity of the prolyl tRNA pool. Rather, in skin from STZ-induced diabetic rats, CMT-1 appeared to act pretranslationally in the collagen biosynthetic pathway, since steady state levels of pro α1(I) collagen mRNA from the diabetic + CMT-1 group were not significantly different from those of non-diabetic or non-diabetic + CMT-1 groups. Furthermore, the effect of CMT-1 on steady state levels of pro $\alpha 1(I)$ collagen mRNA correlated with levels of collagen protein synthesis.

Insulin is known to stimulate levels of pro $\alpha 1(I)$ collagen mRNA and protein synthesis, and insulin removal rapidly returns pro $\alpha 1(I)$ collagen mRNA and protein synthesis to basal levels, presumably through decreased collagen gene transcription and mRNA half life [28]. In the present study, levels of pro $\alpha 1(I)$ collagen mRNA from skin of diabetic rats was 30% of that of non-diabetic controls. However, the administration of CMT-1 to non-diabetic rats did not increase steady state levels of pro $\alpha 1(I)$ collagen mRNA while, in a separate in vitro study, the addition of CMT-1 did not result in increased myoblast protein synthesis [18]. The daily administration of CMT-1 did prevent the decrease in steady state levels of pro $\alpha 1(I)$ collagen mRNA associated with STZ-induced diabetes over the 28-day study period and is in agreement with prior reports of minocycline normalization of skin collagen synthesis and ribosomal mass in STZ-induced diabetic rats [12]. Therefore, the anabolic effect of CMT-1 on skin collagen synthesis in the STZ-induced diabetic rat appeared mediated through the inhibition of depressed steady state levels of pro $\alpha 1(I)$ collagen mRNA, effected perhaps, in part, through prolonged pro $\alpha 1(I)$ collagen mRNA half life.

Two other studies have reported tetracycline-induced changes in steady state message levels. The addition of doxycycline or CMT-1 to keratinocyte cultures after 24 h decreased protein levels of gelatinases MMP-2 and MMP-9. The decreased expression of MMP-2 was associated with decreased hybridizable levels of MMP-2 mRNA by Northern blot analysis and an alteration in cell shape from flattened to spherical, suggesting a loss of cell–cell or cell-matrix contacts with tetracycline addition. It was proposed that doxycycline and CMT-1 may directly decrease MMP-2 mRNA at a proximal site through alteration in rates of MMP-2 transcription or MMP-2 mRNA degradation. It was also proposed that doxycycline and CMT-1 may act indirectly at the cell membrane by decreasing MMP-2 mRNA, through chelation of divalent cations required for cell adhesion molecules, with subsequent changes in gene expression mediated through signal transduction pathways [32].

Evidence for the former, direct role of tetracyclines in the regulation of gene expression has come from a report of depressed steady state stromelysin (MMP-3) mRNA message levels by tetracycline in cultured keratinocytes after MMP-3 induction by interleukin-1 (Il-1) [33]. Decreased steady state levels of MMP-3 mRNA were correlated with decreased rates of MMP-3 transcription, since a plasmid construct consisting of the MMP-3 promoter ligated to the bacterial chloramphenicol acetyl transferase (CAT) coding sequence, when transfected into human skin fibroblasts, resulted in a dose-dependent decrease by tetracycline in MMP-3 transcription after induction of MMP-3 expression by Il-1. Inhibition of MMP-3 expression by tetracycline was found to be independent of the AP-1 domain, however, it was noted that tetracycline alone was not able to depress MMP-3 expression below constitutive levels [33].

Support for an indirect, signal transduction-mediated effect of tetracyclines on steady-state message levels has come from reports that protein kinase C mediated phosphorylation is inhibited in a dose-dependent fashion by tetracyclines [34] and that protein kinase C constitutes a major signal transduction pathway in skin keratinocytes [35]. Furthermore, protein kinase C phosphorylation has been implicated in poly(A) tail removal and subsequent mRNA decay for TNF-α mRNA [36]. Protein kinase C has been demonstrated to both shorten or prolong message half lives for other mRNA as well (reviewed in Ref. [37]). These reports suggest that many of the pleiotropic effects tetracyclines exert, such as alteration in steady state mRNA levels, may act through divalent cation dependent processes including calcium-calmodulin dependent signal transduction pathways [38].

It is also possible that tetracyclines may exert some of their anabolic effects on skin collagen synthesis through a systemic factor. Since a close correlation between body weight and skin collagen synthesis was found in this and in earlier studies [11,12], tetracyclines have been proposed to stimulate the synthesis or release of a general systemic anabolic factor, such as insulin like growth factor 1 (IGF-1) [12]. The present study did not monitor serum levels of IGF-1, however, recent studies have shown that tetracyclines may effect metabolic processes important to diabetic complications. In this regard, excess production of cytokines and prostaglandin E_2, upregulation of inducible nitric oxide synthase and non-enzymatic glycosylation of collagen and other long-lived proteins may all contribute to collagen loss through decreased collagen synthesis and/or increased collagen degradation during diabetes. In addition, these metabolic processes have all been found to be effected by tetracyclines and CMTs [4,39,40].

However, it is clear from the results of the present and related studies [6,11–13,18,20] that tetracyclines function at several sites in the collagen biosynthetic pathway to inhibit connective tissue wasting associated with STZ-induced diabetes. These include the inhibition of extracellular matrix metalloproteinases and the stimulation of skin collagen synthesis through mechanisms independent of the antimicrobial properties of tetracyclines. To effect such disparate cellular functions, it would appear probable that a common biochemical mechanism, such as calcium dependence, could be a common factor. If this hypothesis is correct, the opportunity arises to design future tetracycline derivatives that can inhibit specific divalent cation dependent pathologic processes without the concomitant selection of antibiotic resistant microbial species.

Acknowledgements

This investigation was supported in part by USPHS research grants DE 03318 (M.S.), R37 DE03987 (L.G.) and DE 10094 (R.C.) from the National Institutes of Dental Research, National Institutes of Health, Bethesda, MD.

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